J Assist Reprod Genet (2013) 30:353–359
DOI 10.1007/s10815-013-9937-9

GAMETE BIOLOGY

Oocyte maturation and in vitro hormone production in small antral follicles (SAFs) isolated from rhesus monkeys

Marina C. Peluffo · Jon D. Hennebold · Richard L. Stouffer · Mary B. Zelinski

Received: 21 November 2012 / Accepted: 20 January 2013 / Published online: 20 February 2013

Abstract
Purpose The small antral follicles (SAFs) from the ovarian medulla can be a potential source of oocytes for infertility patients, but little is known about their ability to yield mature oocytes. This study evaluated the response of these SAFs to a stimulatory bolus of human corionic gonadotropin (hCG) in vitro.
Methods Oocyte nuclear maturation and hormone production (estradiol [E2], progesterone [P4]), antimullerian hormone [AMH]) by individual intact SAFs (n=91; >0.5 mm; n=5 monkeys) was evaluated after 34 h of culture in the absence (control) or presence of hCG.
Results Of the total cohort (n=91), 49 % of SAFs contained degenerating oocytes. The percentage of healthy oocytes able to reinitiate meiosis to the metaphase I (MI) and MII was greater (p<0.05) after hCG compared to controls. E2, P4 and AMH levels were higher (p<0.05) in SAF cultures containing germinal vesicle (GV) oocytes compared to those with MII oocytes regardless of hCG exposure. SAF with MI oocytes produced more E2, but less (p<0.05) P4 and AMH compared to SAFs containing GV oocytes (p< 0.05). Follicles ≥1 mm produced more (p<0.05) E2, whereas follicle diameter did not correlate with P4 or AMH levels. Only P4 increased (p<0.05) in response to hCG, regardless of follicle size or oocyte maturity. SAFs containing degenerating oocytes produced similar levels of E2, P4 and AMH compared to SAFs containing healthy oocytes.
Conclusions These data indicate, for the first time, that oocytes within primate SAFs can reinitiate meiosis in vitro in the absence of hCG, but nuclear maturation is enhanced in SAFs cultured with hCG. Oocyte nuclear maturation within SAFs in is associated with decreased E2, P4 and AMH levels. Furthermore, hormone content within the culture media does not necessarily reflect oocyte quality.

Keywords Small antral follicle · Oocyte maturation · Rhesus monkey · E2 · P4 and AMH

Capsule Oocyte nuclear maturation within primate SAFs is enhanced in the presence of hCG, although hormone content in the culture media does not necessarily reflect oocyte quality.

M. C. Peluffo · J. D. Hennebold · R. L. Stouffer · M. B. Zelinski (✉)
Division of Reproductive and Developmental Sciences, Oregon National Primate Research Center, Oregon Health & Science University West Campus, Beaverton, OR 97006, USA
e-mail: mzelinski@ohsu.edu

J. D. Hennebold · R. L. Stouffer
Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, OR 97239, USA

Present Address:
M. C. Peluffo
Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Hospital de Niños Ricardo Gutierrez, Gallo 1330, C1425EFD, Ciudad Autónoma de Buenos Aires, Argentina

Introduction

Fertility preservation in women has become an important issue worldwide and different approaches have been proposed [15]. Cryopreservation of the ovarian cortex is one of the experimental options for restoring fertility that is currently offered to patients in some countries [8]. Even though transplantation of cryopreserved ovarian cortical strips has been successfully performed in some patients [7, 21], in other cancer patients this approach has the risk of introducing cancer cells back [20, 25]. Moreover, this technique only preserves the preantral follicles present within the cortex, but there are many small antral follicles (SAFs) in the ovarian medulla typically not used for cryopreservation and therefore discarded. This cohort of SAFs could be a latent source of oocytes for infertility patients, but little is known about their physiology and potential. The key process required for growing follicles to yield healthy mature oocytes is the acquisition of developmental competence that includes the ability of the

oocyte to resume and complete meiosis, as well as to support embryonic development after fertilization. We recently reported that SAFs collected at any stage of the menstrual cycle (early follicular phase, luteal phase or unknown, i.e., from necropsies) were able to provide cumulus-oocyte complexes (COCs) with oocytes that resume and complete meiosis in vitro to the MII stage, fertilize, and undergo early embryonic development [24]. We also showed that macaque oocytes within COCs derived from healthy SAFs of at least 0.5 mm in diameter can reinitiate meiosis in vitro [23]. However, a better understanding of the SAF and oocyte physiology as well as further improvements in their culture are needed to allow for the efficient development of fully competent oocytes.

Culture of secondary follicles, isolated from rhesus macaque ovaries, in a 3-dimesional (3D) system produced antral follicles of ≤1 mm in diameter that were able to support steroidogenesis and meiosis [33, 34, 36], but whether the enclosed oocytes are developmentally competent is still under investigation. Studies using this 3D system with human follicles also demonstrated the support of in vitro development of preantral follicles up to the very early antral stage, reaching an average size of 800 μm in one month [35]. These sizes/stages of antral development are much smaller than the preovulatory follicle in either women, which is around 20 mm in diameter [19], or in rhesus macaques is up to 6 mm [16]. Moreover, the time interval for folliculogenesis between rodents and primates is quite different. Therefore, there are several technical challenges and hurdles for culturing primate/human follicles relative to rodent that need to be overcome.

The ovary is a reservoir of a large pool of competent GV-stage oocytes that represented an untapped source for female fertility preservation [4]. Further research is warranted to fully define the potential of the cohort of SAFs as another clinical option for fertility preservation. Because of limitations with regard to sample collection as well as ethical issues related to performing experiments in women, the nonhuman primate provides a powerful model to investigate novel sources of competent gametes for fertility preservation. Therefore, studies were designed to characterize and evaluate the response of individual intact SAFs from spontaneously cycling female rhesus macaques to a stimulus of human corionic gonadotropin (hCG) in vitro; including oocyte nuclear maturation and hormone production (estradiol [E2], progesterone [P4]), antimullerian hormone [AMH]).

Materials and methods

Animals

The general care and housing of rhesus monkeys (Macaca mulatta) at the Oregon National Primate Research Center (ONPRC) was previously described [32]. The studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the ONPRC Animal Care and Use Committee.

An initial set of adult female rhesus monkeys (n=6; at the early follicular phase (EFP), average age 8.6 years old) was used to only assess the nuclear oocyte maturation in the presence of hCG using SAFs with or without enclosure in an alginate matrix [33]. But, since no differences were observed between the follicles with or without enclosure in an alginate matrix (Table 1), the majority of the study was performed without an alginate matrix enclosure.

A separate set of animals (n=5; average age 6.4 years old), including those at the early follicular (n=3), or unknown (n=2, from necropsies) phase of the menstrual cycle, were used. The first day of menses was considered day 1 of the cycle. Ovaries were removed from anesthetized monkeys by laparoscopy, as previously described [9]. The excised ovaries were transported immediately to the laboratory in holding media (SAGE®, CooperSurgical, Inc., Trumbull, CT, USA) at 37 °C supplemented with 0.1 % SPS (Serum Protein Substitute, CooperSurgical) plus 10 μg/ml gentamicin (Sigma-Aldrich, St Louis, MO, USA).

SAF isolation and culture

Based on our previous results [23], only SAFs which measured 0.5 mm or greater were used in the present study. Follicle isolation from the ovarian medulla was performed under a dissecting scope using 30 gauge needles. Isolated SAFs (n=91, from 5 animals) were individually cultured for 34 h in the presence or absence of hCG (200 IU/ml; Merck Serono, Geneva, Switzerland) in a 48-well plates containing 300 μl alpha minimum essential medium (αMEM, Invitrogen) supplemented with 0.3 % (v/v) SPS, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Sigma-Aldrich), 220 mIU/ml of recombinant human (rh) FSH (NV Organon, Oss, Netherlands), and 0.5 mg/ml purified bovine fetuin (Sigma-Aldrich).

Table 1 Proportion of healthy oocytes at each stage of nuclear maturation after 34 h in culture with hCG, from either non encapsulated (Control); or encapsulated (Alginate bead) SAFs

Treatment group	GV	MI	MII	MI + MII
Control (n=22)	7/22 (32)*	7/22 (32)	8/22 (36)	15/22 (58)
Alginate (n=11)	5/11 (46)	2/11 (18)	4/11 (36)	6/11 (54)

There were no significant differences (p>0.05) between treatment groups for any of the different maturation stage (GV, MI and MII) oocytes

GV Germinal Vesicle, *MI* Metaphase I, *MII* Metaphase II

*Numbers in parentheses associated with oocyte nuclear maturation data represent the percentage of the different oocyte maturation stages in each group

At the end of culture, the medium from each well was collected, centrifuged and stored at −80 °C for subsequent analysis of sex steroids and AMH [33].

Oocyte maturation

At the end of the culture, COCs were retrieved from the SAFs and treated briefly with (2 mg/ml) hyaluronidase in TALP Hepes-BSA (0.3 %) as previously described [23]. Denuded oocytes were assessed by light microscopy to analyze their nuclear maturation stage (germinal vesicle [GV]-intact; metaphase I [MI] and metaphase II [MII]) or to identify degenerating oocytes (vacuolated, dead).

Hormonal levels (E2, P4 and AMH)

E2 and P4 levels from the culture media were analyzed by the Endocrine Technology Support Core at the ONPRC using an Immulite 2000, a chemiluminescence-based automatic clinical platform (Siemens Healthcare Diagnostics, Deerfield, IL, USA) validated for macaque follicle culture media as reported previously [34]. A commercially available enzyme-linked immunosorbent assay (ELISA) kit was used to detect and quantify the level of AMH protein in the media following the manufacturer's instructions (Diagnostic Systems Laboratories, Inc.) as previous described [33].

Statistical analyses

All statistical calculations were performed using Sigma Stat software package (Systat Software, Inc., Richmond, CA, USA). Differences in hormone content among groups were analyzed using a two-factor analysis of variance (ANOVA) to analyze if they were affected not only by the treatment (hCG vs. Control) but also by oocyte maturation stage or follicle diameter, followed by comparison among means using the Holm-Sidak method. *T*-test was performed when analyzing differences in hormone levels between Control and hCG groups in media from SAFs containing degenerating oocytes. If normality failed, data was transformed prior performing any test. Fisher test was used to analyze differences in proportions in the nuclear maturation rates between Control and hCG groups, or Control vs. Alginate. Differences were considered significant at $p<0.05$.

Results

Oocyte maturation

Table 1 depicts the percentage of nuclear maturation from the healthy oocytes ($n=33$, 6 animals) from the SAFs cultured in the presence of hCG, encapsulated or not in alginate. No significant differences ($p>0.05$) were found between the control (no alginate) and the 3D alginate culture in any of the maturation oocyte stages. In both culture systems, similar percentages of MII oocytes (36 %) were obtained in comparison to the subsequent results showed in Table 2 (32 %). Also, comparable percentages were observed in the MI stages. Although we carefully dissected what appeared to be healthy SAFs, 51 % of oocytes ($n=34$) within the total number ($n=67$) of COCs collected contained vacuolated or dead oocytes at the end of culture.

Oocyte nuclear maturation (detailed in Table 2) was evaluated within the healthy cohort of COCs ($n=46$, 5 animals) retrieved from the SAFs after the end of the culture. The percentage of healthy oocytes resuming maturation to MI and continuing meiosis to MII was higher in the hCG treatment group (32 % for MI and MII, each vs. 11 % and 15 % in the control group; $p<0.05$). In contrast, the highest percentage ($p<0.05$) of GV oocytes was observed in the control group compared to oocytes exposed to hCG (74 % and 36 %, respectively). Regarding vacuolated or dead oocytes, 49 % ($n=45$) of the total COCs ($n=91$) retrieved belonged to this category.

In vitro hormone production

Hormone levels were analyzed according the culture conditions (Control vs. hCG, no alginate) plus follicle size (divided into 2 categories: < 1 mm and ≥1 mm) or oocyte maturation stage to evaluate a possible interaction among these factors (Fig. 1). Higher levels of E2, P4 and AMH were observed in the media from SAFs containing GV oocytes in comparison to those containing MII oocytes ($p<0.05$). SAFs with MI oocytes produced higher levels of E2, but lower levels of P4 and AMH in comparison to the SAFs with GV oocytes ($p<0.05$). While the larger follicle diameter correlated with higher levels of E2 in the culture media ($p<0.05$), follicle size did not affect the levels of P4 or AMH. The hCG treatment did not have a significant effect on E2 or AMH levels. In contrast, P4 levels increased in hCG group ($p<0.05$) regardless of follicle

Table 2 Proportion of healthy oocytes at each stage of nuclear maturation retrieved from SAFs at the end of the culture, in either the control or hCG group

Treatment Group	GV	MI	MII	MI + MII
Control ($n=27$)	20/27 (74)*[a]	3/27 (11)[a]	4/27 (15)[a]	7/27 (26)[a]
hCG ($n=19$)	7/19 (36)[b]	6/19 (32)[b]	6/19 (32)[a]	12/19 (64)[b]

Different letters represent significance differences ($p<0.05$) between treatment groups for GV, MI and MII stage oocytes, respectively

GV Germinal Vesicle, *MI* Metaphase I, *MII*, Metaphase II

*Numbers in parentheses associated with oocyte nuclear maturation data represent the percentage of the different oocyte maturation stages in each group

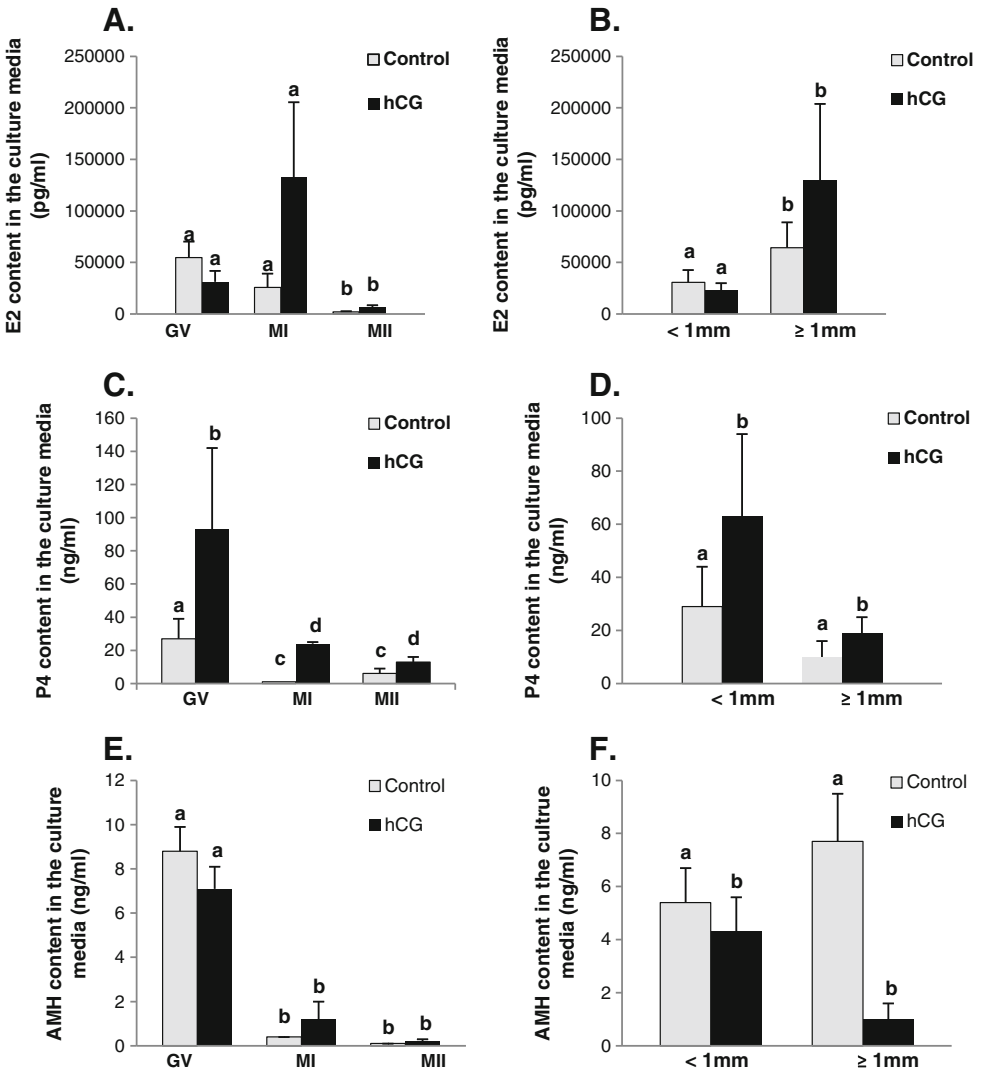

Fig. 1 Steroids (E2 and P4) and AMH levels in the culture media produced by rhesus macaque SAFs (according to the stage of oocyte nuclear maturation or follicle size) after 34 h of culture in the presence (Control) or absence of hCG (hCG). Panels **a**, **c**, **e** represent hormone values according treatments and oocyte nuclear maturation stage. Panels **b**, **d**, **f** represent hormone values according treatments and follicle size. Values are the mean ± SEM of SAFs collected from 5 animals, and represent 25 (Control) and 17 (hCG) number of follicles per group. Different letters represent significant differences among time points ($p<0.05$)

size or oocyte maturation stage. No interactions were observed between the treatment and any of the two factors analyzed (follicle size and oocyte nuclear maturation). However, treatment and follicle size tended to have an interaction in regards to AMH levels ($p=0.064$).

E2, P4 and AMH levels from representative media from SAFs containing degenerating oocytes ($n=26$) were analyzed (Fig. 2). All samples from both groups (Control, $n=13$ and hCG, $n=11$) exhibited significant levels of these hormones that were similar to those produced by healthy oocytes (see Fig. 1). Most notably, the SAFs containing degenerating oocytes were able to respond to the hCG treatment with increased progesterone production (see Fig. 2, Panel b).

Discussion

The present results demonstrate that primate SAFs are able to respond in vitro to an ovulatory stimulus, promoting the resumption and progression of meiosis. However, this response is much lower in comparison to its counterparts during isolated macaque COC culture, 32 % vs. 48–82 % [23, 24]. Moreover, a small proportion of SAFs without a stimulus spontaneously reinitiated meiosis to the MI or MII stage, suggesting that SAFs can enclose oocytes at different stages of maturation. In our laboratory, while dissecting SAFs from non-stimulated ovaries, the presence of one or two oocytes at the MII stage was frequently observed within these follicles (data not shown). Moreover, the COCs from these oocytes exhibited expanded cumulus complexes noticeable by the enlargement and stickiness of their matrix. Sirard proposed that the process of early atresia mimics several of the pre-ovulatory changes that may be are perceived by the oocyte as pre-ovulatory signals [26], such as the rise in progesterone and androgen, as well as the progressive decrease of follicular support from the granulosa layer. Interestingly, the percentages of degenerating oocytes retrieved from the SAF population after 34 h of culture are

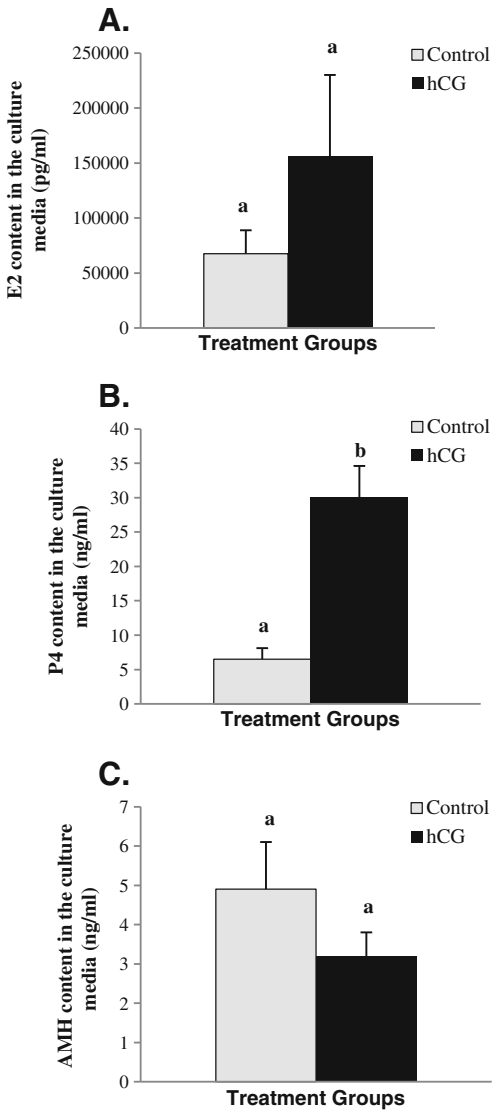

Fig. 2 Hormone contents (Panel **a**: E2, Panel **b**: P4, Panel **c**: AMH) in the culture media produced by rhesus macaque SAFs enclosing degenerating oocytes. Values are the mean ± SEM from representative samples from each treatment group (Control, n=13 and hCG, n=11). Different letters represent significant differences among time points (p<0.05)

in accordance with our previous results observed when using and isolating follicles from fresh tissue [23, 24]. Thus, this degeneration cannot be linked to the culture itself but rather to the health of the SAFs. It seems that at this early stage of the antral follicle, some of the follicles/oocytes have been already selected to die. The cohort of SAFs present within the ovarian medulla is known to be more susceptible to atresia [17]. Also, the majority of this growing pool of follicles will never get to the preovulatory stage and instead they will suffer atresia [13].

When comparing culture systems (no alginate or alginate) in the presence of hCG, similar percentages of MII oocytes were obtained, showing that the alginate bead does not interfere with hCG action. Nevertheless, 3D culture does not improve the outcomes, either. Many recent efforts have been made to culture preantral follicles up to the preovulatory stage in different species. Live births have been achieved in rodents using different systems of in vitro follicle culture [5, 6, 11, 12, 18, 28]. However, success has not yet been achieved in larger animals. The technical challenges of follicle culture in larger animals (including primates and women) with longer time interval during folliculogenesis as well as preovulatory follicle size relative to mice are obvious. Our laboratory has been working on developing and improving culture conditions for 3D culture system of primate preantral follicles [33–36]. However, SAFs developed in vitro (of around 1 mm in diameter) from encapsulated 3D culture of macaque secondary follicles were able to respond to an hCG stimulus promoting the resumption of meiosis up to the MII stage in only around 10 % of the treated follicles [33]. So, the main question/-concern is how to follow the follicle growth and development as well as which parameters should be measured without disturbing the culture. Researchers have been commonly measured hormone content in vitro culture, especially steroids, as a parameter of follicle health and growth [27, 30, 33–36]. Also, steroid production has been used as a parameter of health/function after cryopreservation of ovarian tissue [14, 29]. However, do steroids actually reflect the oocyte quality or health?

To respond to this question, hormone levels were measured in the culture media of SAF in the presence or absence of hCG. Our results showed that hCG treatment increased P4 levels in the culture media of SAFs regardless of follicle size or oocyte maturation stage, showing that at this early developmental stage the follicles are able to respond to the hCG stimulus. But, no interactions were observed between hCG treatment and follicle size or oocyte nuclear maturation. However, this response is not physiological since in vivo these small follicles are not supposed to respond to the LH surge. A premature response to LH of the granulosa cells from human SAFs (4–8 mm) from anovulatory women with PCOS was reported, in comparison to their counterparts from normal or ovulatory PCOS women [31]. On the other hand, higher levels of E2 in the culture media correlated with greater follicle diameter. Most likely due to the increase in number of granulosa cells able to secrete E2 in the larger diameter SAFs. In contrast, follicle size did not affect the levels of P4 or AMH, suggesting that the number of granulosa cells is not correlated to the amount of P4 and/or AMH synthesized and/or secreted. Also, E2 and AMH levels were not significantly affected in the presence of hCG. The reason for the decrease in AMH levels in the media from SAFs with a diameter ≥1 mm can be explained by the fact that all the enclosed oocytes were at MI or MII stage. In addition, treatment groups were analyzed according oocyte maturation stage, significant differences were not observed with hCG treatment.

An interesting finding was that the highest levels of E2, P4 and AMH were observed in the media from SAFs containing GV oocytes. It seems that the hormone levels (E2, P4 and AMH) within the follicle need to be low in order to contain an MII oocyte. Whether this decrease occurs before/during meiotic progression or as a consequence of this process remains to be determined. We can speculate that at a certain point of the follicle development the oocyte takes the lead and somatic cell activities decrease. This idea is in accordance with the concept of several researchers that the oocyte controls follicular functions and development [1, 3, 10]. It was shown that AMH was a good predictor of the ovarian response and gonadotropin-dose adjustment, but a poor predictor of embryo quality and pregnancy chances in individual patients [2], suggesting that this hormone levels may predict quantity but not quality. On the other hand, AMH inhibits factors affecting FSH sensitivity and its overproduction in anovulatory polycystic ovaries may therefore restrict folliculogenesis by an inhibitory effect on FSH sensitivity, thereby contributing to anovulation [22]. It is important to consider that the media from SAFs containing degenerating oocytes exhibited considerable levels of all these hormones (E2, P4 and AMH). Most notably, they were able to respond to hCG treatment in vitro. Collectively, the content of these hormones cannot be used as a marker of follicle/oocyte health since they would probably only reflect the health or amount of granulosa/somatic cells present in the follicle. However, hormonal production does not reveal the characteristics (such as number, balance, communication, health, etc.) of the different cell populations present in the follicle during folliculogenesis. Eppig has hypothesized that in the absence of the oocyte from the follicle, gonadotrophins and factors from the theca cells would drive the development of all granulosa cells toward the mural granulosa cell phenotype and ultimate luteinization [10]. A major limitation of current research in follicle culture as well as in vitro maturation is the lack of assays for appropriate biomarkers of oocyte health.

In summary, these data indicate that oocytes within primate SAFs in vitro are able to reinitiate meiosis in the absence of hCG, but nuclear maturation is enhanced in SAFs cultured in the presence of hCG. Oocyte nuclear maturation within SAFs in vitro is associated with lower levels of E2, P4 and AMH relative to GV oocytes. However, there is an increase in P4 in response to hCG. Whether these decreases occur before/during or are a consequence of meiotic progression remains to be determined. Furthermore, hormone content within the culture media does not necessarily reflect oocyte quality and cannot be used as a marker of follicle/oocyte health in this type of culture, since SAFs containing degenerated oocytes produce high hormone (E2, P4 and AMH) levels and are hCG-responsive with respect to P4 production. Novel markers to assess oocyte growth, maturation and quality culture during follicle culture are still warranted.

Acknowledgments We are grateful for the expert contributions of the animal care staff and surgical unit of the Division of Animal Resources, and the technical support of the ART core and the Endocrine Services and Technology Laboratory at ONPRC.

Supported by Oncofertility Consortium NIH 1 UL1 RR024926 (R01-HD058294, PL1-EB008542), the Eunice Kennedy Shriver NICHD Specialized Cooperative Centers Program in Reproduction and Infertility Research (U54 HD18185), the Eunice Kennedy Shriver NICHD Contraceptive Development and Research Center (U54 HD55744), NCRR-RR000163, NIH R01 HD052909 (MC) and a Lalor Foundation Postdoctoral Basic Research Fellowship (MCP).

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